Applied and Environmental Microbiology, May 2006, p. 3245–3251 Vol. 72, No. 5
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.5.3245–3251.2006

Characterization of Shiga Toxin Gene (*stx*)-Positive and Intimin Gene (*eae*)-Positive *Escherichia coli* Isolates from Wastewater of Slaughterhouses in France

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Received 22 November 2005/Accepted 21 February 2006

Wastewater samples from 12 slaughterhouses located in different regions in France were tested for the presence of *stx*-positive and *eae*-positive *Escherichia coli* isolates, and characteristics of the isolates obtained were determined. A total of 224 wastewater samples were collected in wastewater treatment plants at different stages of wastewater processing. Altogether, 5,001 *E. coli* isolates were obtained by colony counting and screened for the presence of *stx* and *eae* genes by multiplex PCR. *stx*-positive and *eae*-positive *E. coli* isolates were detected in 25% of the samples collected; they were found in 13% and 3% of the samples obtained from treated effluent and sludge, respectively, suggesting that they could be spread into the environment. Screening of the samples collected by immunomagnetic separation allowed us to isolate 31 additional *E. coli* serogroup O157 isolates. Four of these isolates harbored *stx* and *eae* genes. All *stx*-positive and *eae*-positive *E. coli* isolates were analyzed for *eae* and *stx* genetic variants, as well as for additional virulence factors and serotypes. Our results suggest that the majority of the *stx*- and *eae*-positive *E. coli* isolates from wastewater have low virulence for humans. However, the diversity of the enterohemorrhagic *E. coli*-associated virulence factors in the strains indicates that the environment may play an important role in the emergence of new pathogenic enterohemorrhagic *E. coli* strains.

Enterohemorrhagic *Escherichia coli* (EHEC) is a recent emerging group of food-borne pathogens. Since 1982, EHEC strains have caused worldwide outbreaks of hemorrhagic colitis which have led in 10% of the cases to life-threatening hemolytic-uremic syndrome (HUS) (7).

E. coli of serotype O157:H7 is the leading cause of EHEC infections in humans, but other EHEC serotypes, such as O26:H11 or O26:H–, O103:H2, O111:H–, O113:H21, and O145:H28 or O145:H–, have been increasingly associated with outbreaks and sporadic cases of EHEC-associated disease in humans (3, 7). The reason certain serotypes are associated with disease in humans has not been fully elucidated. The inventory and precise functions of EHEC virulence factors are still not known, but epidemiological studies have suggested that associations between virulence factors could increase the ability of some serotypes to cause disease in humans (5). Certain *E. coli* strains (Shiga toxin [Stx]-producing *E. coli* [STEC]) produce Shiga toxins that are encoded by *stx* genes and damage intestinal, vascular, and renal cells (20). *stx* genes are carried by lysogenic bacteriophages and can be acquired by horizontal gene transfer (25, 26). Therefore, it has been suggested that the emergence of EHEC strains has resulted from successive horizontal transfers of virulence factors between STEC and enteropathogenic *E. coli* (EPEC) (7). Stx production is essential but not sufficient for EHEC virulence. The majority of EHEC strains produce a characteristic histopathological feature at the microvillus brush border of enterocytes of humans, known as an "attaching and effacing" (A/E) lesion, by subverting the intestinal epithelial cell function. The formation of A/E lesions is governed by a pathogenicity island known as the locus of enterocyte effacement (LEE), which contains the *eae* (*E. coli* attachment effacement) gene encoding the intimin protein (7). The presence of the *eae* gene correlates highly with the presence of other genes of the LEE (1) in EHEC strains, as well as in EPEC strains. The majority of EHEC strains harbor both *stx* and *eae* genes, but some *eae*-negative STEC strains, such as O91:H21 and O113:H21 strains, which cause bloody diarrhea and HUS in humans, have been described previously (7).

A wide range of animal species are known to carry STEC and EHEC strains, but ruminants are the most important natural reservoir and excrete these bacteria with their feces (7). Although the main infection routes are person-to-person transmission, as well as consumption of contaminated meat and milk, ingestion of contaminated vegetables or water and direct contact with animals or soil have also been associated with EHEC-associated outbreaks. It has been reported that human infections can result from ingestion of fewer than 100 viable EHEC cells (7). Moreover, EHEC and STEC can persist and remain infectious for several weeks in slurries, farmyard manure, and sewage sludge, as well as on pasture land (10, 17, 23). Land application of sludge and effluent discharge in surface water contribute to the spread of STEC in the environment. Contamination of the environment, followed by uptake of STEC by farm animals on pasture, maintains the epidemiological cycle of STEC and is a public health concern. In

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TABLE 1. Pathotypes, serotypes, and virulence factors of reference strains used for genotypic and phenotypic analysis of *E. coli* strains isolated from slurry, wastewater, and river water collected in and near French slaughterhouses

Strain	Pathotype (species[a])	Serotype	Virulence factors screened	Accession no. or reference[b]
MG 1655	*E. coli* K-12[c]	Orough:H48		U00096
RIMD 050992	EHEC (human)	O157:H7	*eae* (conserved region), stx_1, stx_2 (conserved region)	16
B2F1	EHEC (human)	O91:H21	$stx_{2vh\text{-}a}$, $stx_{2vh\text{-}b}$, stx_2 G1, *saa*[d]	31
CF11201	EPEC (human)	O125:H–	*eae*-η	38
CL37	EHEC (human)	O111:H8	*eae*-θ	38
H-19	EHEC (human)	O26:H11	*eae*-β_1	29
E 2348/69	EPEC (human)	O127:H6	*eae*-α_1, *bfpA*	29
EDL 933	EHEC (human)	O157:H7	*eae*-γ_1, $stx_{2\text{-}EDL933}$, *ehxA*, *toxB*, $fliC_{h7}$	29
ICC95	EPEC (human)	O86:H34	*eae*-β_2/δ	29
PMK5	EHEC (human)	O103:H2	*eae*-ε, stx_1	29
S1191	STEC (pig)	O139:H–	stx_{2e}	2
EF73	EPEC (human)	O125:H6	*eae*-α_2	29
95NR1	Nonpathogenic *E. coli*	O111:H–	*eae*-γ_2	29
4795/95	STEC (human)	O84:H4	*eae*-ζ	38
6044/95	EHEC (human)	O118:H5	*eae*-κ	38
7476/97	EHEC (human)	O145:H4	*eae*-ι	38

[a] Species from which the strain was isolated.
[b] Place where the strain's virulence factors were first described.
[c] Laboratory strain.
[d] The stx_2 G1 group defined by Nakao et al. (27) contains the stx_{2c}, stx_{2cf}, $stx_{2\text{-}OX3b}$, $stx_{2vh\text{-}a}$, $stx_{2vh\text{-}b}$, $stx_{2vh\text{-}c}$, $stx_{2vh\text{-}c(Lin)}$, $stx_{2vh\text{-}d}$, and AB017524 subtypes.

addition, data on the presence and characteristics of STEC in urban and slaughterhouse wastewater are limited and difficult to compare (11, 12, 14, 18, 22, 37).

The aim of this study was to determine the presence and characteristics of *stx*-positive and *eae*-positive *E. coli* strains in slurry, wastewater, and river water collected in and near slaughterhouses located in different parts of France. Detection and characterization of virulence genes, together with serotyping of *stx*- and *eae*-positive *E. coli* strains and analysis of the function of LEE-encoded genes, allowed us to estimate the significance of these organisms for public health. The combination of the different virulence factors found mainly on mobile genetic elements and the different phenotypic characteristics allowed us to determine the diversity of *E. coli* strains better. Altogether, our results helped us to determine the role of the environment in the emergence of new pathogenic STEC and EHEC strains.

MATERIALS AND METHODS

Sample description. A total of 224 samples of slurry, wastewater, and river water were collected in and near 12 slaughterhouses in France. Samples were obtained from each slaughterhouse seasonally between February 2002 and September 2003. Six slaughterhouses located in the west of France (Bretagne and Normandie regions) each had a complete wastewater treatment plant and released their effluents into rivers. Six slaughterhouses located in the southwest of France (Aquitaine and Midi-Pyrénées regions) did not have a complete wastewater treatment plant and passed their effluents to an urban wastewater treatment plant. Wastewater samples were collected at different points during wastewater treatment. Two liters of raw wastewater (40 samples), 2 liters of screened wastewater (40 samples), and 2 liters of primary treated wastewater (34 samples) were collected from each slaughterhouse. Samples of slurry (35 2-liter samples) and sludge (29 2-liter samples) intended to be spread on land were also collected. Samples of treated wastewater intended to be released into rivers were obtained only from slaughterhouses with complete wastewater treatment plants (16 2-liter samples). River water samples were collected upstream (15 5-liter samples) and downstream (15 5-liter samples) of the effluent discharges of slaughterhouses that released their effluents into rivers.

Isolation of *E. coli*. Briefly, decimal dilutions of the 224 homogenized samples collected were plated on selective medium for *E. coli* (Petrifilm Select Coli; BioMérieux, Marcy l'Etoile, France) and cultured at 44°C for 24 h. For each sample, 20 to 24 CFU were selected at random, biochemically confirmed to be *E. coli* with an API 20E test (BioMérieux), and grown separately with agitation overnight at 37°C in Luria-Bertani broth (Invitrogen, Paisley, Scotland). Broth cultures of each isolate were stored at −80°C in 30% sterile glycerol.

To improve the sensitivity of detection of *E. coli* O157, a complementary isolation procedure was performed. Aliquots of the 224 homogenized samples were enriched in modified Trypticase soy broth at 42°C for 16 h as described previously (9). Immunomagnetic separation of *E. coli* O157 was performed according to the manufacturer's instructions (anti-*E. coli* O157 Dynabeads; Dynal Biotech, Bromborough, United Kingdom). The beads were plated on sorbitol MacConkey agar (Oxoid, Hampshire, United Kingdom) and cultured at 37°C for 24 h. Presumed *E. coli* O157 strains were confirmed by biotyping with the API 20E test (BioMérieux) and by latex agglutination (*E. coli* O157 test kit; Oxoid) with non-sorbitol-fermenting isolates from the plates.

Reference strains. Broth cultures of all reference strains listed in Table 1 were stored at −80°C in 30% sterile glycerol. Each strain was grown with agitation overnight at 37°C in Luria-Bertani broth.

Laboratory nonpathogenic *E. coli* strain MG1655 was used as a negative control for all virulence factors investigated. EHEC O157:H7 strain RIMD 050992 (Sakaï) was used as a positive control for detection of the stx_1, stx_2 (conserved region), and *eae* (conserved region) genes by multiplex PCR. EHEC, EPEC, and STEC strains listed in Table 1 were used as positive controls for detection of other virulence factors and for typing and subtyping of the *eae* and stx_2 genes. *E. coli* strains CL37, 95NR1, 4795/95, 6044/95, and 7476/97 were kindly provided by H. Schmidt, Stuttgart, Germany. *E. coli* strain EF73 was kindly provided by S. Morabito, Rome, Italy.

Laboratory nonpathogenic *E. coli* strain MG1655 was used as a negative control for the fluorescence actin staining (FAS) test. EPEC O127:H6 strain E2348/69 and EHEC O103:H2 strain PMK5 were used as positive controls for the FAS test.

Detection of virulence genes by PCR. DNA from the isolates of *E. coli* was subjected to multiplex PCR for detection of the virulence genes *eae*, stx_1, and stx_2 by using combinations of the eaeA B52, eaeA B53, sltI B54, sltI B55, sltII B56, and sltII B57 specific primers as described previously (8). The amplification products were analyzed by electrophoresis on a 2% agarose gel.

Plasmid-borne virulence genes were screened by PCR. The presence of the enterohemorrhagic *E. coli* hemolysin (EHEC-HlyA) encoded by the *ehxA* gene was investigated as described previously (33). The genes encoding the bundle-forming pili (*bfpA*), the STEC autoagglutinating adhesin (*saa*), and the ToxB adhesin (*toxB*) were investigated as previously described (15, 30, 34). PCR amplification products were analyzed by electrophoresis on 1.5% agarose gels.

Variants of the B subunit of stx_2 genes. Subtypes of the B subunit of stx_2 genes were determined by PCR, followed by analysis of restriction endonuclease-digested PCR products (restriction fragment length polymorphism [RFLP]) as described previously (19, 32). $stx_{2\text{-EDL 933}}$, $stx_{2vh\text{-}a}$, $stx_{2vh\text{-}b}$, $stx_{2d\text{-}Ount}$, and $stx_{2d\text{-}OX3a}$ variants were differentiated by PCR using the VT2-e and VT2-f primers, followed by digestion with HaeIII and PvuII of the PCR amplification products (32). The presence of variant stx_{2e} was investigated by PCR using primers Vte-a and Vte-b as described previously (19). However, these methods failed to detect some of the stx_2 variants that are present in patient isolates, and Nakao et al. (27) have described a multiplex PCR to detect the most prevalent stx_2 variants in EHEC strains classified as variants $stx_{2\text{-EDL 933}}$, stx_{2ent}, and $stx_{2\text{-}O48}$ and group stx_2-G1 corresponding to the variants stx_{2c}, stx_{2cf}, $stx_{2\text{-}OX3b}$, $stx_{2vh\text{-}a}$, $stx_{2vh\text{-}b}$, $stx_{2vh\text{-}c}$, $stx_{2vh\text{-}c(Lin)}$, and $stx_{2vh\text{-}d}$, and an unnamed stx_2 variant (GenBank accession no. AB017524). Variants $stx_{2\text{-EDL 933}}$, stx_{2ent}, and $stx_{2\text{-}O48}$ were investigated by using combinations of primers mStx2, G1-F, and mG1-R primers, and group stx_2-G1 was investigated by using combinations of primers mStx2, G1-F, and mStx2-R, as described previously (27). The amplification products and restriction analysis products were examined by electrophoresis on 1% agarose gels.

Types and subtypes of intimin genes. Eight intimin types (α, β, γ, ε, ζ, η, ι, and κ) were investigated by PCR using combinations of forward primer SK1 and reverse primers LP2 to LP10, as described previously (38). To further differentiate the different types of intimin genes, PstI restriction analysis of the PCR amplification products was performed to differentiate α_1, α_2, β_1, β_2/δ, γ_1, and γ_2/θ variants, as described previously (29). The amplification products and restriction analysis products were examined by electrophoresis on 1% agarose gels.

FAS test with HeLa cells. Expression of LEE-encoded genes leading to A/E lesions was tested by visualization of filamentous actin accumulation at sites of bacterial adhesion on cultured human epithelial cells. The FAS test was performed by using a protocol described previously (29).

Serotype identification. The O antigens of all isolates that had *eae*, stx_1, and stx_2 genes were determined with O antisera specific for the 11 most prevalent O antigens detected in EHEC strains (O26, O48, O91, O103, O111, O113, O118, O128, O145, O146, and O157) according to information found at the Laboratorio de Referencia de *E. coli* website (http://www.lugo.usc.es/ecoli/SEROTIPOSHUM.htm). The H types of motile isolates that belonged to one of the serogroups screened were analyzed by serotyping using antisera specific for 53 different H antigens (H1 to H56). Nonmotile isolates were investigated to determine their H-type-specific (*fliC*) genes by PCR, followed by HhaI digestion of *fliC* PCR products and evaluation of RFLP patterns, as previously described (4).

All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove nonspecific agglutinins. The O antisera were provided by the Laboratorio de Referencia de *E. coli* (Lugo, Spain), and the H antisera were obtained from the Statens Serum Institute (Copenhagen, Denmark). Additional O and H typing of *E. coli* strains was performed at the National Reference Laboratory for *Escherichia coli* at the Federal Institute for Risk Assessment, using O and H antisera produced at the Federal Institute for Risk Assessment.

RESULTS

Presence of *stx*-positive and *eae*-positive *E. coli* isolates. A total of 224 samples of slurry, wastewater, and river water were collected in and near 12 slaughterhouses in France in different seasons and at different points in connected wastewater treatment plants. Altogether, 5,001 isolates of *E. coli* were obtained and screened for the presence of *eae*, stx_1 and stx_2 genes by multiplex PCR.

Twenty-five percent (55/224) of all samples contained at least one *E. coli* isolate carrying *eae*, stx_1, and stx_2 genes. The positive samples were found to be equally distributed in all of the slaughterhouses investigated. However, one slaughterhouse was more contaminated with *stx*-positive and *eae*-positive *E. coli* (80% of the samples were contaminated). The high contamination rate was later explained by malfunctioning and shutting down of the wastewater treatment plant. Characteristics of samples containing *stx*-positive and *eae*-positive *E. coli* are shown in Table 2. The total numbers of *E. coli* organisms were determined for all samples and were found to be similar for all sampling points independent of the season and slaughterhouse. In general, samples from early stages of wastewater treatment (raw, screened, and primary treated wastewater samples) were more contaminated with *E. coli* and with *stx*-positive and *eae*-positive types than samples from the final stages (effluent thrown out and effluent sludge) were. Nevertheless, *stx*-positive and *eae*-positive *E. coli* isolates were detected in all kinds of effluent that were released into the environment (treated effluents flowing into rivers, sludge and slurry intended to be spread on land, and primary treated wastewater flowing into urban wastewater treatment plants). In general, river water collected upstream from the slaughterhouses was less contaminated than river water collected downstream.

TABLE 2. Characteristics of wastewater samples containing *stx*- and *eae*-positive *E. coli* isolates, as determined by colony counting

Sampling point in slaughterhouse wastewater treatment plant	No. of samples tested	No. (%) of samples containing: STEC[a]	*stx*-negative, *eae*-positive *E. coli*	Total
Slurry	35	4 (11)	6 (17)	10 (29)
Raw wastewater	40	5 (13)	12 (30)	14 (35)[b]
Screened wastewater	40	5 (13)	7 (18)	12 (30)
Primary treated wastewater	34	5 (15)	5 (15)	10 (29)
Effluent thrown out to river	16	0	2 (13)	2 (13)
Sludge	29	0	1 (3)	1 (3)
River water upstream from effluent discharge	15	2 (13)	0	2 (13)
River water downstream from effluent discharge	15	2 (13)	2 (13)	4 (27)
Total	224	23 (10)	35 (16)	55 (25)[b]

[a] Including *eae*-positive STEC.
[b] Three samples of raw wastewater contained both STEC and *stx*-negative, *eae*-positive *E. coli*.

Characterization of *stx*-positive *E. coli* isolates. Thirty independent isolates of *stx*-positive *E. coli* were detected among the 5,001 *E. coli* isolates by colony counting. These 30 STEC isolates were considered to be independent since they were obtained from different samples or (when they were isolated from the same sample) had different virulence factors or biotypes, as determined by the API biochemical code.

The Shiga toxin types and variants of the stx_2 and *eae* genes of the 30 STEC isolates were determined by PCR and PCR-RFLP analysis and are shown in Table 3.

Except for O128, most of the STEC isolates did not belong to the 11 serogroups frequently associated with EHEC strains.

The STEC strains were heterogeneous for the *stx* genes; 14 strains harbored only stx_1, and 15 strains harbored only stx_2. One STEC strain contained both stx_1 and $stx_{2\text{-}vhb}$. Three stx_2-positive strains harbored more than one stx_2 gene. The most frequent stx_2 variant was stx_{2e} (detected in six STEC strains), followed by $stx_{2vh\text{-}a}$ or $stx_{2vh\text{-}b}$ (detected in three STEC strains) and $stx_{2\text{-EDL933}}$ (detected in two STEC strains). One stx_2-positive STEC strain had an stx_2 variant that belonged to group 1 as defined by Nakao et al. (27), which is the most important stx_2 variant group in terms of human pathogenicity. Five stx_2-positive STEC strains contained stx_2 genes which were nontypeable with the methods described above.

Most of the STEC isolates were *eae* negative; only two *eae*-positive STEC strains were detected, and they were associated with intimin β_1.

Characterization of *eae*-positive *E. coli* isolates. It is known that Shiga toxin gene-negative (*stx*-negative) and intimin gene-positive (*eae*-positive) strains of *E. coli* can acquire *stx* genes by

TABLE 3. Variants of *stx* and *eae* genes for the most frequent EHEC serogroups detected for 30 independent STEC isolates by colony counting in samples of slurry, wastewater, and river water collected in and near French slaughterhouses[a]

Serogroup[b]	Type and/or subtype(s) of *stx* gene	Intimin variant	No. of independent STEC isolates
O128	stx_1		2
	$stx_{2vh\text{-}a}$ and $stx_{2vh\text{-}b}$	*eae*-β_1	1
ND[c]	stx_1	*eae*-β_1	1
	stx_1		11
	$stx_{2vh\text{-}b}$ and stx_1		1
	$stx_{2vh\text{-}a}$ and $stx_{2vh\text{-}b}$		1
	stx_2 G1 different from $stx_{2vh\text{-}a}$ and $stx_{2vh\text{-}b}$[d]		1
	$stx_{2\text{-}EDL933}$		1
	$stx_{2\text{-}EDL933}$ and stx_{2e}		1
	stx_{2e}		5
	$stx_{2\ NT}$[e]		5

[a] The STEC isolates included *eae*-positive STEC.
[b] Only the 11 most prevalent O antigens associated with EHEC strains were screened (O26, O48, O91, O103, O111, O113, O118, O128, O145, O146, and O157).
[c] ND, not determined.
[d] stx_2 gene belonging to group 1 defined by Nakao et al. (27) but different from the $stx_{2vh\text{-}a}$ and $stx_{2vh\text{-}b}$ subtypes. The stx_2 subtype could correspond to subtype stx_{2c}, stx_{2cf}, $stx_{2\text{-}OX3b}$, $stx_{2vh\text{-}c}$, $stx_{2vh\text{-}c(Lin)}$, or $stx_{2vh\text{-}d}$ or to an unnamed subtype (GenBank accession no. AB017524).
[e] $stx_{2\ NT}$, nontypeable variant of the stx_2 gene.

horizontal transfer, increasing their pathogenicity for humans (25, 26). The present work provided new information because we studied not only STEC isolates but also *stx*-negative, *eae*-positive *E. coli* isolates. On the other hand, *stx*-negative, *eae*-positive strains of *E. coli* could be derivatives of STEC strains that have lost their phage-encoded *stx* genes (24).

Fifty-four independent *stx*-negative, *eae*-positive *E. coli* isolates were detected among the 5,001 *E. coli* isolates by colony counting. The intimin types and intimin variants of these isolates were determined by PCR and PstI RFLP analysis (Table 4).

TABLE 4. Intimin types and variants for the most frequent EHEC serogroups detected for 54 independent *stx*-negative, *eae*-positive *E. coli* isolates by colony counting in samples of slurry, wastewater, and river water collected in and near French slaughterhouses

Intimin variant	Serogroup[a]	No. of independent *stx*-negative, *eae*-positive *E. coli* isolates
β_1	O26	3
	O103	3
	ND[b]	7
β_2/δ	ND	2
γ_1	O145	1
	ND	11
γ_2/θ	ND	18
κ	ND	2
η	ND	1
ζ	ND	1
NT[c]	O157	1
	ND	4

[a] Only the 11 most prevalent O antigens associated with EHEC strains were screened (O26, O48, O91, O103, O111, O113, O118, O128, O145, O146, and O157).
[b] ND, not determined.
[c] NT, nontypeable variant of the *eae* gene.

Most of the *stx*-negative, *eae*-positive *E. coli* strains did not belong to the 11 serogroups frequently associated with EHEC. The most frequent EHEC-related serogroups detected were O26 and O103, followed by O145 and O157.

Neither intimin α, intimin ε, nor intimin ι was detected in any isolate. The most frequently found intimin was intimin γ_2/θ (detected in 18 isolates), followed by intimin β_1 (detected in 13 isolates) and intimin γ_1 (detected in 12 isolates). Six isolates harbored intimin β_2/δ, κ, η, or ζ. Five isolates carried intimin variants different from the 11 variants screened.

All *stx*-negative, *eae*-positive *E. coli* isolates were *bfp* negative and could therefore be classified as "atypical EPEC" (28).

Detection of *E. coli* O157 isolates. *E. coli* O157 STEC strains were not present among the 5,001 *E. coli* strains isolated from wastewater samples. This collection of *E. coli* isolates was probably representative of the dominant populations of *E. coli* and did not take into account nondominant populations of *E. coli* present in slaughterhouse wastewater. In order to screen specifically for *E. coli* O157, O157 immunomagnetic separation enrichment followed by a latex agglutination assay with sorbitol-negative *E. coli* isolates was performed for all samples. In this analysis, 31 *E. coli* O157 strains were isolated and screened for the presence of *eae*, stx_1, and stx_2 genes by multiplex PCR. Variants of the *eae* and stx_2 genes were screened by PCR and PCR-RFLP analysis. Four *E. coli* O157 isolates were positive for intimin γ_1, and two of these harbored both $stx_{2vh\text{-}a}$ and $stx_{2vh\text{-}b}$ variants. These four isolates belonged to the O157:H7 serotype.

Characterization of the putatively most virulent *E. coli* isolates. All *stx*-positive *E. coli* strains and *stx*-negative, *eae*-positive *E. coli* strains isolated from slaughterhouse wastewater were characterized further in order to assess their significance for public health.

E. coli strains belonging to serotypes O26:H11 or O26:H–, O103:H2, O111:H–, O113:H21, O145:H28 or O145:H–, and O157:H7 are recognized as classical EHEC types (3). In addition, apart from their capacity to produce Shiga toxins and intimins, most EHEC strains have accessory virulence factors, such as enterohemolysin (EHEC-HlyA), which seems to be associated with severe clinical disease (5, 33). Furthermore, intestinal adherence factors distinct from intimin, such as Saa and ToxB, play a role in the virulence of some *E. coli* strains by increasing the capacity for adhesion (31, 35).

Detection of these additional virulence factors, together with determination of the complete serotype, allowed us to select 13 isolates which belonged to serotypes associated with illness in humans (Table 5). Most of these isolates were detected in the early stages of wastewater treatment, but four isolates (two serotype O128:H8 STEC isolates and two *stx*-negative, *eae*-positive serotype O26:H11 or O157:H7 *E. coli* isolates) were detected in wastewater released into the environment. Three *eae*-positive STEC isolates, including one O128:H8 isolate and two O157:H7 isolates, harbored more than three different virulence factors that are particularly associated with EHEC strains. The last two STEC isolates belonged to serotype O128:H8 and were negative for *eae*, but they harbored a potential adhesion factor encoded by the *saa* gene. Seven of the eight *stx*-negative, *eae*-positive isolates of *E. coli* that belonged to a serotype associated with human illness

TABLE 5. Phenotypes, genotypes, and origins of the 13 putatively most virulent *E. coli* isolates from slurry, wastewater, and river water collected in and near French slaughterhouses

Putatively virulent *E. coli* isolate	Phenotype		Genotype						Sampling point
	Serotype[a]	FAS activity	*eae*	stx_2	stx_1	*ehxA*	*saa*	*toxB*	
STEC[b]	O128:H8[c]	+	β_1	$stx_{2vh\text{-}a}$, $stx_{2vh\text{-}b}$	–	–	–	–	Primary wastewater flowing into urban wastewater treatment plant
	O128:H8[c]	NA[d]	–	–	+	–	+	–	Slurry
	O128:H8[c]	NA	–	–	+	–	+	–	Raw wastewater
	O157:H7I[e,f]	–	γ_1	$stx_{2vh\text{-}a}$, $stx_{2vh\text{-}b}$	–	+	–	+	Raw wastewater
	O157:H7[e,f]	–	γ_1	$stx_{2vh\text{-}a}$, $stx_{2vh\text{-}b}$	–	+	–	+	Raw wastewater
stx-negative, *eae*-positive *E. coli*	O26:H11[e]	+	β_1	–	–	–	–	–	Primary wastewater flowing into urban wastewater treatment plant
	O26:[H11][e]	+	β_1	–	–	–	–	–	Raw wastewater
	O26:[H?][e]	+	β_1	–	–	–	–	–	Raw wastewater
	O103:H2[e]	+	β_1	–	–	–	–	–	Raw wastewater
	O145:[H28][e]	+	γ_1	–	–	–	–	+	Raw wastewater
	O157:H7[e]	–	NT[g]	–	–	–	–	–	Raw wastewater
	O157:H7[e,f]	+	γ_1	–	–	+	–	+	Screened wastewater
	O157:H7[e,f]	+	γ_1	–	–	+	–	+	Effluent thrown out

[a] Only O:H serotypes of *E. coli* isolates that belonged to a serotype associated with illness in humans are included. An H type in brackets indicates the presence of nonmotile strains, which were analyzed to determine their *fliC* types by PCR as described in Materials and Methods. H? indicates the presence of an H type different from H1 to H56.
[b] Including *eae*-positive STEC.
[c] Serotype of *E. coli* which has frequently been associated with EPEC strains (13) but has never been associated with EHEC strains.
[d] NA, not applicable.
[e] Serotype of *E. coli* which has frequently been associated with EHEC strains (3).
[f] *E. coli* isolates from nondominant populations of *E. coli* serotype O157 from French slaughterhouse wastewater.
[g] NT, nontypeable variants of the *eae* gene.

contained an intimin variant frequently associated with HUS, four isolates that belonged to serotype O26:H11, O26:H– or O103:H2 were positive for β_1 intimin, and three serotype O145:[H28] or O157:H7 isolates harbored γ_1 intimin. The last *stx*-negative, *eae*-positive isolate, which belonged to serotype O157:H7, harbored a nontypeable variant of the *eae* gene.

The ability of *eae*-positive isolates of *E. coli* to produce A/E lesions on cultured HeLa cells was determined by the FAS test. Eight of 11 *eae*-positive strains which belonged to serotypes associated with illness in humans induced accumulation of filamentous actin at sites of adhesion.

DISCUSSION

One aim of the present study was to determine the presence of *stx*-positive and *eae*-positive *E. coli* isolates in slurry, wastewater, and river water collected in and near 12 French slaughterhouses.

Twenty-five percent of the samples collected were contaminated with *stx*-positive and *eae*-positive *E. coli* isolates, and positive samples were found to be equally distributed in all of the slaughterhouses studied. These results show that potentially pathogenic *E. coli* isolates could be found ubiquitously in French slaughterhouses and are in agreement with the results of a previous study of contamination of the environment with STEC in France (37).

Higher numbers of *stx*- and *eae*-positive *E. coli* isolates were detected in samples from early stages of wastewater treatment than in samples from the final stages of wastewater treatment, confirming the results of a previous report on reduction of STEC in wastewater after treatment (12). Thus, wastewater treatment could be appropriate for reducing the survival of STEC and *eae*-positive *E. coli*, as it is for reducing *E. coli* viability. Nevertheless, the total numbers of *E. coli* organisms in samples could not be considered a reliable indicator of the presence of STEC and *eae*-positive *E. coli*. Indeed, some *stx*-positive and *eae*-positive *E. coli* isolates were detected in sludge samples that contained relatively low numbers of *E. coli* organisms, whereas *stx*-positive and *eae*-positive *E. coli* isolates were not detected in some samples of raw wastewater that contained high numbers of *E. coli*. Some *stx*-positive and *eae*-positive *E. coli* isolates were detected in slaughterhouse wastewater that was ready to be released into the environment. Moreover, river water collected upstream from the slaughterhouses was less contaminated than river water collected downstream from the slaughterhouses. These results suggest that STEC and *eae*-positive *E. coli* can persist during all stages of wastewater treatment and are able to adapt to environmental stress in aquatic systems and survive, thus confirming results of a previous study of the resistance of *E. coli* O157:H7 to routine water treatment procedures (21). Persistence of STEC in wastewater during treatment has been observed previously in France (37), Germany (18), and Tasmania (22). However, it was difficult to compare the numbers of STEC isolates since the isolation procedures were different. Nevertheless, the results indicate that contamination of natural water sources can occur directly or indirectly from the regular disposal of sewage, although this possibility was once rejected (14). As a consequence, our results indicate that the environment is a reservoir for human-pathogenic *E. coli* isolates, which has consequences for public health.

All STEC and *stx*-negative, *eae*-positive *E. coli* isolates originating from wastewater released by slaughterhouses were characterized further in order to assess their potential as possible human pathogens.

Although some stx_1-positive STEC isolates have previously been associated with severe disease (6), epidemiological studies have demonstrated that Stx2 is the most important virulence factor associated with severe human disease (5, 20) and is significantly associated with an increased risk of HUS in persons infected with O157 or non-O157 STEC (6). In the present study, one-half of the STEC isolates (15/30) harbored stx_2 genes and can thus be considered to be more closely associated with an increased risk of HUS than the stx_1-positive STEC isolates.

Twenty stx_2 variants have been identified on the basis of sequence homology, and at least 12 stx_2 variants produced by EHEC strains from patients have been described previously (2, 27). Some other variants, which are associated with STEC strains isolated from specific hosts, such as sheep (stx_{2d}) and pigs (stx_{2e}), are considered to be less pathogenic for humans (7). The type of *stx* variant could thus reflect not only the origins and relationships but also the virulence of the different STEC strains. In our collection of environmental STEC isolates, the most frequent stx_2 variants were stx_{2e} and nontypeable variants of stx_2, which are probably less pathogenic for humans. The stx_{2e} variants were isolated from samples collected in slaughterhouses in which pigs are slaughtered, a finding which is in agreement with previously published data (7). However, stx_2 variants produced by EHEC strains, such as $stx_{2vh\text{-}a}$, $stx_{2vh\text{-}b}$, $stx_{2\text{-}EDL933}$, and variants of the G1 group, which was defined by Nakao et al. (27) as the most important stx_2 variant group in terms of human pathogenicity, were detected in STEC isolates. Our results indicate that very diverse stx_2 variants are associated with STEC isolates, confirming the results of previous findings for STEC strains isolated from the environment in France (36). The majority of STEC strains probably originated from livestock, but lysogenization of *E. coli* strains that harbor stx_2 variants produced by EHEC strains may lead to conversion of new strains of *E. coli* and to the emergence of new human pathogens (25).

The presence of the *eae* gene in EHEC strains is significantly associated with an increased risk of bloody diarrhea in humans (6), and at present 11 variants of intimin have been described previously (38). The distribution of these variants among EHEC and EPEC strains isolated from different species suggests that the host and/or the tissue tropism of the different A/E bacteria may be influenced by the variants of intimin that they express. In our collection of *E. coli* isolates, the majority of *eae*-positive isolates can be considered "atypical EPEC" and are probably characteristic of animal strains, which could be explained by the nature of the samples collected. Indeed, intimin α, which seems to be specifically expressed by human classical EPEC strains (29), was not found in any *eae*-positive *E. coli* isolate. Furthermore, even if the majority of *eae*-positive *E. coli* isolates harbored intimins γ_2/θ, β_1, and γ_1, which are expressed by human strains (29, 38), most of them did not belong to serogroups frequently associated with EHEC strains and were probably less pathogenic for humans. In addition, intimin variants that are probably rare among strains associated with severe human disease, such as intimins β_2/δ, κ, η, and ζ, or intimin variants that are different from those screened (38) were detected in *eae*-positive isolates. The presence of these nontypeable intimins could be explained by the frequency of recombination events inside the LEE and especially inside the *eae* gene (38). Thus, the great diversity of intimin variants, including those associated with EHEC strains, detected in our environmental collection of *eae*-positive *E. coli* isolates indicated that the environment could play a role as a reservoir for pathogenic strains and for recombination of intimin genes (38).

Based on the intimin and Stx variants and serotypes, most of the *stx*-positive and *eae*-positive *E. coli* isolates were probably low-virulence strains, which confirmed the results of a previous characterization of STEC strains isolated from Spanish urban sewage and animal wastewater (11). The majority of *E. coli* isolates in our study had serotypes and combinations of virulence genes that are not frequently associated with hemorrhagic colitis or HUS, and they were probably more specific *E. coli* strains present in animal digestive microfloras. These isolates may nonetheless play an important role in the assortment of genes between *E. coli* isolates, which could lead to the emergence of new pathogenic EHEC strains. In addition, 13 strains putatively virulent for humans were also detected. The presence of such virulent isolates could have been underestimated since decimal dilution of the samples collected allowed isolation of strains that were primarily representative of the dominant *E. coli* populations. Indeed, STEC serotype O157:H7 isolates were present in the nondominant *E. coli* populations, as demonstrated by the immunomagnetic separation procedure.

In conclusion, this study demonstrated that slurry, wastewater, and river water present in and near French slaughterhouses are contaminated with STEC and with *eae*-positive *E. coli* and highlights the importance of appropriate handling and use of slurry and sewage sludge to prevent contamination of the environment and food by EHEC. In addition, the great diversity of the *stx*- and *eae*-positive *E. coli* isolates from wastewater of slaughterhouses suggests that monitoring the flux of EHEC virulence factors in the bacterial population in the environment is necessary to prevent epidemiological risks for public health.

ACKNOWLEDGMENTS

This study was supported by the research program "Environnement et santé 2000 INSERM-contrat no. EN00C04 RD: AC006G" from the Ministère Français de l'aménagement du territoire et de l'environnement.

We thank E. Sandrin-Gabriel-Robez from the Biogram Society for her technical assistance.

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